RADIOIMMUNOASSAYS FOR ANDROSTERONE, 5α-ANDROSTANE-3α,17β-DIOL AND 5α-ANDROSTANE-3β,17β-DIOL

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ABSTRACT

Androsterone (3α-hydroxy-5α-androstan-17-one), 5α-androstane-3α, 17β-diol and 5α-androstane-3β,17β-diol were conjugated at C-16 through sulfur to bovine and human serum albumin. Rabbits injected with these conjugates produced antibodies suitable for radioimmunoassays of these hormone metabolites. Samples were purified on Sephadex LH-20 columns. Levels of these steroids were measured in a rat blood serum pool and in ovarian tissue extract pools.

INTRODUCTION

There have been numerous reports in recent years implicating the androgen metabolites 5α-androstane-3α,17β-diol (3α-A-diol) and 5α-androstane-3β,17β-diol (3β-A-diol) in physiological regulation of gonadotropin secretion (1-3) and of the onset of puberty (4-6). These interpretations have been based on pharmacological studies in which exogenous steroids were administered to animals and on measurement of levels of these steroids in peripheral serum, using tedious and insensitive methods of analysis (4-6). In order to facilitate further study of their physiological importance, we have raised antisera against these steroids and against androsterone and have utilized these antisera for the development of specific radioimmunoassay (RIA) methods suitable for measurement of picogram amounts of these steroids in biological samples. In this paper we report these methods and their utilization in measurement of levels of the three steroids (3α-A-diol, 3β-A-diol and

androsterone) in rat serum pools and in rat ovarian extracts.

RIA methods for measuring androsterone (7,8) and for 5α-androstane diols (8-10) have been recently described, but they involve complicated synthetic work in order to obtain suitable steroid-albumin conjugates or long chromatography procedures to separate 3α-A-diol from 3β-A-diol. We have used Pang and Johnson's method (11) for preparation of conjugates through bromo derivatives to bovine and human serum albumins (BSA and HSA), which have been enriched with sulfhydryl groups by the method of Klotz and Heiney (12). Hapten prepared by this method had good antigenic properties and produced, when injected into rabbits, antibodies with reasonable titres and specificities.

MATERIALS AND METHODS

Solvents and reagents:

All solvents and reagents were of analytical grade; solvents were purchased through Fisher Scientific Co., and were redistilled prior to use. Bovine serum albumin (BSA) (fraction V), human serum albumin (HSA), S-acetyl-mercaptosuccinic anhydride and thimerosal were bought from Sigma Co.; silica gel G-254 from Brinkmann Co.; Sephadex LH-20 from Pharmacia; androsterone, 3α-A-diol, 3β-A-diol, and 3β-hydroxy-5α-androstan-17-one (epiandrosterone) were obtained from Steraloids, Inc.; complete Freund's Adjuvant from Difco Co.; (^{3}H 1,2) androsterone, specific activity 40-60 Ci/mmole, (^{3}H 1,2) 3α-A-diol, specific activity 40-60 Ci/mmole, and (^{3}H 1,2) 3β-A-diol, specific activity 40-60 Ci/mmole were obtained from New England Nuclear Corporation.

Animals:

New Zealand white rabbits were used for the production of antisera. Sprague-Dawley rats were purchased from Bio-Breeding Laboratories, Ottawa, Canada.

Antigen preparation:

a) Steroids

16α-bromo-3α-hydroxy-5α-androstan-17-one (16α-bromoandrosterone) and 16α-bromo-3β-hydroxy-5α-androstan-17-one (16α-bromoepiandrosterone)

Androsterone or epiandrosterone (290 mg, 1 mmole) and 1.275 x 10^6 DPM of tritiated hormone (in the case of androsterone) were refluxed

(13) in 25 ml of chloroform. Bromine (160 mg, 1 mmole) in 5 ml of $CHCl_3$ was added dropwise. Fifteen minutes after decolorization the reaction was stopped, 30 ml of water added, and extracted 2 x with 30 ml of $CHCl_3$. Organic layers were washed with 30 ml of 1 N sodium bicarbonate and 30 ml of water, combined and dried over anhydrous $MgSO_4$, concentrated *in vacuo* and applied on four 20 x 20 cm, 1 mm thick thin layer chromatographic (TLC) plates, and developed in a 30% ether-benzene system. Compounds were visualized under U.V. light or by spraying side strips of TLC with 10% H_2SO_4 and subsequently by burning with a hot glass rod. The bromo derivatives (14-18) were recovered as crystalline compounds (30%). Sixteenα-bromo-3α-hydroxy-5α-androstan-17-one was recrystallized from acetone-heptane (m.p. 196-200°C). Mass spectra (19) of trimethylsilylether derivative (TMSi) was characteristic for bromo compounds by producing doublets at m/e 440 and 442 (M^+), m/e 425 and 427 [M^+-15] and m/e 350, 352 [M^+-90] and also contained major fragments at m/e 306, m/e 271 [M^+-(90+80)], m/e 75 (base peak). Infra red spectra ($CHCl_3$) 3600 cm^{-1}, 3400 cm^{-1} (broad peak), 1750 cm^{-1}. Sixteenα-bromo-3β-hydroxy-5α-androstan-17-one was recrystallized from acetone-heptane (m.p. 154-156.5°C). Mass spectra of TMSi derivative contained same fragments which were slightly different from 3α-epimer in relative intensities of several major fragments. IR ($CHCl_3$) 3600 cm^{-1}, 3400 cm^{-1} (broad), 1750 cm^{-1}.

16α-bromo-5α-androstane-3α,17β-diol and 16α-bromo-5α-androstane-3β,17β-diol

Sixteenα-bromoandrosterone or 16α-bromoepiandrosterone (100 mg, 0.271 mmole) were dissolved in 20 ml of methyl alcohol and reduced with 30% excess of sodium borohydride (20) at room temperature for 3 h. The reagent was decomposed by an addition of 1 ml 0.1 N HCl. After another hour 25 ml of water was added and bromo diols were extracted into 3 x 30 ml of chloroform. The combined organic layers were dried over anhydrous $MgSO_4$, concentrated and purified on preparative TLC (see above); 80% of oily bromo 3α-A-diol and 85% of crystalline bromo 3β-A-diol were isolated. Mass spectra of trimethylsilyl derivatives of 3α-A-diol and 3β-A-diol produced molecular ions at m/e 514 and 516 (due to isotope abundance of ^{79}Br and ^{81}Br), m/e 499 and 501 [M^+-15], m/e 434 [M^+-80], m/e 419 [M^+-(80+15)], m/e 344 [M^+-(80+90)], m/e 254 [M^+-(80+90+90)], m/e 129 (base peak). Relative intensities of some fragments were different in both epimers. Infra red spectra were devoid of absorption in 1700 cm^{-1} region and contained broad absorption around 3400 cm^{-1}.

b) Proteins

Sulfhydryl (SH) groups were introduced into BSA and HSA molecules through the reaction of S-acetylmercaptosuccinic anhydride with amino groups of lysine, according to the procedures of Klotz and Heiney (12) and Pang and Johnson (11).

The coupling of bromo steroids to SH-enriched BSA and HSA was done according to Pang and Johnson (11). The incorporation of steroid onto the molecules of proteins was calculated on the basis of radioactivity and was found, in the case of 16α-bromoandrosterone, to be between 15

and 20 residues per molecule of albumin. Incorporation of the 16α-bromo-3α-A-diol and 16α-bromo-3β-A-diol was not tested.

Antibody preparations:

For each antigen three rabbits were immunized with 1 mg of steroid-protein conjugate in 1 ml of 1:1 saline and complete Freund's adjuvant mixture. The emulsion was injected in 5-10 sites intracutaneously and 1-2 sites subcutaneously. Animals were boosted every 2 weeks for 3 months, after which titres were tested 7-10 days after every booster injection. BSA-steroid conjugates were then exchanged for HSA antigens. This procedure resulted in a marked increase in the titre of antiserum against 3β-A-diol after a single boost. Titres reached maximum levels after 4 months of immunizations.

Radioimmunoassay (RIA) procedure:

a) Sample preparation

1) Pooled serum samples from immature female rats (200 µl) were extracted with 3 x 3 volumes of diethylether. About 1000 CPM of tritiated androsterone and 3α-A-diol was added prior to extractions to every tube to assess recovery efficiency of the extraction and consequent Sephadex LH-20 column separation. Combined ether extracts were dried and reconstituted in 200 µl of $CHCl_3$ for applications on columns. For recovery studies 50 and 500 pg of appropriate steroids were added into samples prior to the extraction.

2) Rat ovarian extract pool was prepared by homogenization of 50 rat ovaries in 25 ml of ethyl alcohol. For recovery assessment, radioactive tracer was added into 200 µl aliquots which were taken, dried down in a stream of nitrogen and applied on columns in 200 µl of $CHCl_3$. To study the recoveries of added steroids, 50 and 500 pg of appropriate hormones were added into 200 µl samples before the extractions.

b) Chromatography

Androsterone and epiandrosterone were separated from 3α-A-diol and 3β-A-diol on 23 x 0.6 cm Sephadex LH-20 columns (21) using chloroform: heptane:methyl alcohol:water (500:500:75:3 volumes) solvent system. With a flow rate of approximately 20 ml/h, androsterone was consistently recovered in 5-6 ml of eluate and both diols appeared in the 8-11 ml fractions. Eluates were dried down in a stream of nitrogen and reconstituted in 1 ml of ethyl alcohol, from which aliquots were taken for radioactive recovery measurements and for RIA determinations.

c) Radioimmunoassay

Standard curves ranged from 10-640 pg. Standards were pipetted into tubes in different volumes of ethanol, dried down in a stream of nitrogen and reconstituted in 100 µl of buffer (0.1 M sodium phosphate buffer, pH 7.4; 0.1% gelatin; 0.01% thimerosal). Samples were treated in the same fashion. One hundred microlitres of appropriately diluted antibody (30-50% bound/total (Bo/T) radioactivity) was followed by 100 µl of tritiated steroids (10,000 CPM/tube). Tubes were incubated at

4°C overnight and dextran-coated charcoal was used to separate bound from free hormones (1 ml of 0.25% charcoal and 0.025% dextran per tube). After 15 min incubation and centrifugation at 1000 x *g* at 4°C, supernatants were counted in a toluene-based scintillation solution (5 g PPO in 1 litre of 3.5% glacial acetic acid in toluene), in a Searle β counter. Data were evaluated with the aid of an on-line Searle computer.

RESULTS

Titres

All immunized rabbits developed some antibodies. The three best were chosen according to their titres and specificities. Maximum titres were obtained after 4 months of immunization. For 50% binding (Bo/T) anti-androsterone (rabbit AO-1), anti-3α-A-diol (rabbit AHα-3), and anti-3β-A-diol (rabbit AHβ-3) were diluted 1:1000, 1:800 and 1:5000, respectively.

Crossreactivity

The results of crossreaction studies with 21 steroids are reported in Table 1. The antisera were highly specific for the configuration at C-3 and C-5 but lacked specificity for the configuration at C-17. This necessitated sample purification on Sephadex LH-20 prior to RIA. Androsterone, epiandrosterone and progesterone metabolites (the last of which could interfere with RIA for 3α-A-diol) are eluted prior to 3α-A-diol and 3β-A-diol.

Rat serum and ovarian extract dilutions (parallelism of assays)

Serum pool and ovarian extract pools were extracted, purified as described before and assayed using three different aliquots of each sample. Samples were also fortified with 500 pg of appropriate steroid. Two hundred µl, 100 µl and 50 µl of ethyl alcohol reconstituted samples were assayed. The results when corrected for dilutions were almost identical (see Figures 1-3).

TABLE 1

Percent Crossreaction of Anti-androsterone (AO-1), Anti-5α-androstan-3α,17β-diol (AHα-3) and Anti-5α-androstan-3β,17β-diol (AHβ-3)

Compounds	AO-1	AHα-3	AHβ-3
Cholesterol	< 0.3	< 0.3	< 0.3
3β-hydroxy-5-pregnen-20-one	< 0.3	< 0.3	< 0.3
5α-pregnane-3,20-dione	< 0.3	< 0.3	< 0.3
3α-hydroxy-5α-pregnan-20-one	< 0.3	28.4	< 0.3
3α,17α-dihydroxy-5α-pregnan-20-one	< 0.3	18.3	< 0.3
Cortisol	< 0.3	< 0.3	< 0.3
Corticosterone	< 0.3	< 0.3	< 0.3
Testosterone	< 0.4	< 0.3	1.6
4-androstene-3,17-dione	< 0.4	< 0.3	1.8
17β-hydroxy-5α-androstan-3-one	< 0.4	< 0.3	14.7
5α-androstane-3,17-dione	< 0.4	< 0.3	10.4
Androsterone	100	100	< 0.3
3α-hydroxy-5β-androstan-17-one	< 0.4	< 0.3	< 0.3
3β-hydroxy-5β-androstan-17-one	< 0.4	< 0.3	< 0.3
3β-hydroxy-5α-androstan-17-one	< 0.4	< 0.3	15.2
5α-androstane-3α,17β-diol	27	100	< 0.3
5α-androstane-3β,17β-diol	< 0.4	< 0.3	100
5β-androstane-3α,17β-diol	< 0.4	< 0.3	< 0.3
5β-hydroxy-5-androsten-17-one	< 0.3	< 0.3	< 0.3
Estrone	< 0.3	< 0.3	< 0.3
Estradiol-17β	< 0.3	< 0.3	< 0.3

Recovery

For the determination of recoveries, tritiated steroids were added to serum or ovarian tissue extracts. Aliquots were counted following extraction and purification on Sephadex LH-20. Recoveries for ^{3}H-androsterone, ^{3}H-3α-A-diol and ^{3}H-3β-A-diol were determined to be 86.9 ± 6.0, 93.5 ± 3.7 and 78.1 ± 1.1% (n = 11), respectively, for serum and 95.0 ± 6.2, 92.7 ± 2.2 and 67.3 ± 9.0% (n = 7), respectively, for ovarian tissue.

The rat serum and rat ovarian extract pools which were fortified with 50 and 500 pg of androsterone, 3α-A-diol and 3β-A-diol, respectively, gave results which are summarized in Figures 4-6.

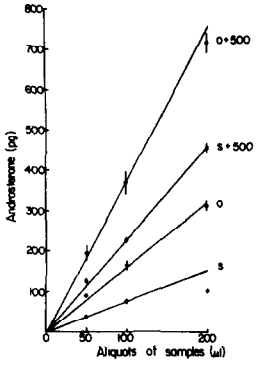

Figure 1

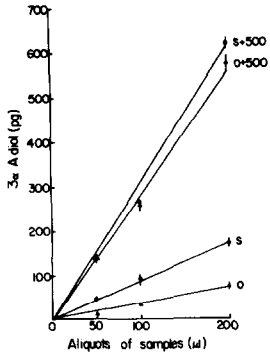

Figure 2

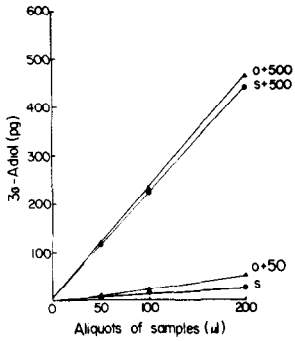

Figure 3

Figures 1-3. Androsterone, 3α-A-diol and 3β-A-diol assay of 50, 100 and 200 µl aliquots of serum and ovarian tissue extract pools and pools into which 500 pg of appropriate steroid was added. In the case of 3β-A-diol ovarian extract, 50 pg of 3β-A-diol was also added, since basal levels in ovarian extracts were below detection limits of the assay (mean ± S.E.M.; n = 4).

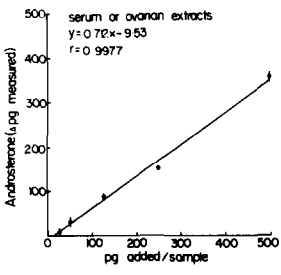

Figure 4

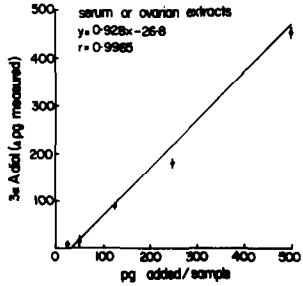

Figure 5

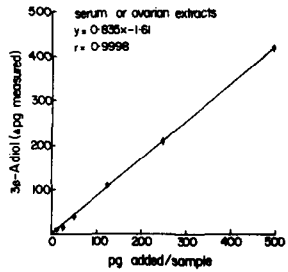

Figure 6

Figures 4-6. Androsterone, 3α-A-diol and 3β-A-diol assay of serum and ovarian tissue extract pools fortified with various amounts of appropriate steroids (Δ pg = pg measured in fortified samples less basal levels found in pools) (mean ± S.E.M.; n = 4).

Sensitivity

Sensitivity is defined by Midgley *et al.* (22) as the smallest amount of non-labelled antigen causing a significant drop of binding of tritium-labelled antigen (i.e., the minimum amount of unlabelled antigen which produces a decrease in binding greater than two standard deviations below that of the zero dose). In the RIA for 3β-A-diol, the minimum amount was 10 pg, and 20 pg for 3α-A-diol and androsterone assays.

All solvents and column eluates were tested for possible blank values and all were found below the sensitivity of the assay.

Precision

Six serum or ovarian tissue extract pools in quadruplicate were extracted, purified on Sephadex LH-20 columns and assayed in three separate assays for all three steroids. The average intra-assay and inter-assay coefficient of variation for androsterone was 16.3% and 18.17%, respectively; 3α-A-diol 14.2% and 16.3%, respectively; and 3β-A-diol 8.6% and 12.6%, respectively.

DISCUSSION

Radioimmunoassays have become one of the most widely used methods of quantification of minute amounts of different compounds in biological samples. Coupling of the steroid hormones to BSA, HSA or other macromolecules and subsequent immunization of animals with these antigens produce specific antibodies which are then utilized for competitive binding of radioactively labelled and unlabelled compounds. Specificities of these antibodies (23) can, to a certain extent, be influenced by the site of the coupling between steroid and macromolecule. If an already present functionality is used for the linkage to protein, antibodies usually will not distinguish conformational changes around these

sites. Ideally the best approach, then, is to use very distant positions on steroid molecules so that more specific antibodies can be obtained. This, in most cases, involved lengthy synthesis since these steroids are not usually available commercially. Our relatively simple approach of using bromoderivatives, which can be easily prepared, and the subsequent connection through the reaction with SH-enriched BSA or HSA produced antigens which gave rise to antibodies with usable titres and specificities.

Chin and Warren (24) reported that bromoderivatives of progesterone can covalently link with the sulfhydryl groups of cystein of 20β-hydroxy-steroid-dehydrogenase. Pang and Johnson (11) reported preparation of 6β-thioprogesterone and 6β-thiotestosterone-BSA conjugates using a similar approach. In our laboratory a 21-thio(3α-hydroxy-5α-pregnan-20-one)-BSA conjugate was prepared and used for the preparation of highly specific antisera against this progesterone metabolite (unpublished observations). This involved an introduction of bromine to the molecule of 3α-hydroxy-5α-pregnan-20-one at C-21 and subsequent reactions of this functional group with sulfhydryl groups of either cystine or cysteine amino acids present in molecules of BSA or HSA. Alternatively, albumins can be enriched with this group by reaction with S-acetylmercapto-succinic anhydride (12). The latter approach was selected since more reactive groups can be obtained. Androsterone or epiandrosterone can be brominated at C-16 and the bromoketones then used for subsequent conjugation. Reduction of the 17-keto group with sodium borohydride (20) yields mostly 17β alcohols while retaining the halogen on the neighbouring carbon. 3α-A-diol or 3β-A-diol can then be conjugated to proteins.

As our studies of crossreactivity showed (Table 1), the resulting antibodies were quite capable of distinguishing changes at C-3 and C-5 of the steroid molecule, but were less effective for compounds differing at C-17. Therefore, some sort of separation had to be applied. We have used the modified method of Murphy (21) since we found that Sephadex LH-20 did not contribute to the assay blank and separation was relatively easy and reproducible with recoveries over 70%. Androsterone, epiandrosterone and progesterone metabolites are separated from 3α- and 3β-A-diol which can then be measured in the same eluate since the antibodies are very specific for them.

The above-described RIA methods were tested extensively on rat samples to establish validity of these assays for our other studies of these important androgens in male (25) and female rats.

ACKNOWLEDGEMENTS

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